THE CHEMICAL NATURE OF IMMUNE SUBSTANCES

MICHAEL HEIDELBERGER

Hospital of the Rockefeller Institute for Medical Research, New York

Immunology, one of the youngest of the biological sciences, has expanded with such rapidity and developed with such intricacy and complexity that an understanding of its chemical basis has lagged far behind. Structural organic chemistry, colloid chemistry and physical chemistry have been freely drawn upon and much data have been accumulated on the chemical, physical and immunological behavior of the substances concerned in the phenomena of immunity. The interpretation of these data is often rendered difficult by the necessarily imperfect methods of isolation of the complex and labile substances involved; hence there is relatively little known of the actual chemical nature of the material with which the immunologist must work.

The chemical basis of immunity has been so adequately presented in Professor Wells' splendid monograph (1) that it would be futile for the writer to attempt a repetition of this task. He would like, however, to present a summary of such knowledge as is available in the narrower field of the actual chemical nature of immune substances, freely acknowledging his indebtedness to Professor Wells' book, and attempting chiefly to cover such advances as have been made since it was written.

Present methods of organic and biological chemistry are such that the separation of a highly active specific substance from generally larger amounts of inert accompanying impurities is an exceedingly difficult matter, and is only possible of realization under particularly favorable conditions of stability on the part of the specific material. For this reason a more detailed treatment is given to the section on Carbohydrates than to those on Proteins and Lipoids. It is felt that on the score of thermostability, inertness to the comparatively brutal reagents often necessary, and a sharply defined specificity which remains qualitatively the same before and after purification, the carbohydrates with immunologically specific properties offer perhaps the best available material for the acquisition of definite knowledge as to the chemical basis of specificity.

Even in this group it is necessary to proceed with extreme caution,

since the substances isolated are amorphous, of high molecular weight, and devoid of the criteria which would stamp them beyond a doubt as pure substances. This is even more forcibly the case in the protein group, with, for example, toxins and antibodies, and in the last analysis the reviewer of this field must deal with trends and tendencies, rather than with completely established proofs.

In a treatment of the subject such as this many papers of immunological interest will necessarily be omitted and only those chosen for discussion which contribute in as reasonably clear-cut a fashion as possible to a broader understanding of the true chemical nature of immune substances.

I. PROTEINS AND PROTEIN DERIVATIVES. *A. General.* Protein chemistry is at present in a period of transition, and there is a tendency to ascribe increasing importance to diketopiperazine (2), oxazoline (3), and possibly even pyrrole ring (4) structures as components of the complex protein molecule, rather than to assume a straight-chain polypeptide skeleton, as Emil Fischer did. This newer conception is already being reflected in immunochemical work, and will be discussed under the head of toxins.

In the light of recent work it still seems quite evident that highly purified proteins may function as antigens. Convincing evidence has been offered that this is also true in the case of oxyhemoglobin (5), hitherto the most doubtful antigen of the more easily accessible crystalline and recrystallizable proteins.

Likewise it still seems clear that the presence of protein or the higher protein degradation products is necessary in order for a substance to function as an antigen. It would appear, also, that the antigenic properties of a protein may be profoundly modified, not only by chemical reaction (as in Landsteiner's classic work), but also in the native state by combination with a lipoid or with a carbohydrate, so that changes in specificity are not always to be traced to changes, subtle or otherwise, in the protein molecule itself. This will become evident in subsequent discussions.
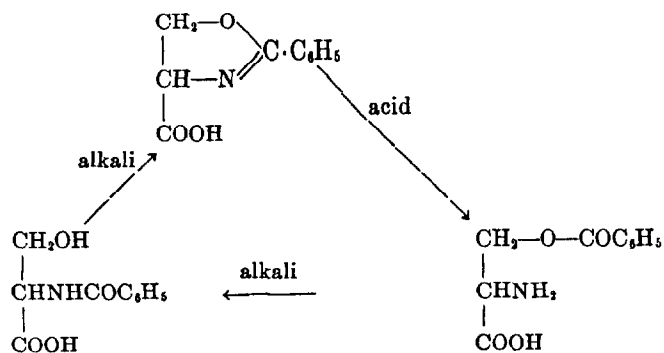
Little can be said as to the actual groupings in the complex protein molecule which determine specificity. Landsteiner has found that among the azoproteins the most sharply defined specificity and least overlapping are shown by those in which the diazotized aromatic component possesses an acid group, such as in the aminobenzoic acids, sulfanilic acid and arsanilic acid. On the basis of this work and the finding of fatty acids in the specific portion of the heterogenetic antigen and sugar acids in the polysaccharides discussed below, Landsteiner

ascribes especial significance to acid groups in the determination of specificity (6).

B. Bacterial toxins. Recent work has tended to confirm the belief that the bacterial toxins belong among the higher protein degradation products. Dernby's studies of diphtheria toxin production and destruction (7) led him to the view that the toxin is not a lipoid, since it is rapidly destroyed by proteolytic enzymes, and that it originates through the action of the proteolytic enzymes of the bacillus on the organism itself and even on the primary proteoses of the medium, the proteolysis ultimately continuing until the toxin is destroyed. Watson and Langstaff (8), who applied Watson and Wallace's modification (9) of Glenny and Walpole's acetic acid precipitation method (10) to formaldehyde-treated diphtheria toxin, found this to be very stable on reprecipitation and dialysis. The purified toxoid reacted as a proteose and contained sulfur.

Huntoon (11) has found that in the purification of scarlatinal toxin addition of 1 per cent of acetic acid and 60 per cent saturation with ammonium sulfate give rise to a voluminous precipitate which carries very little toxin. After removal of this "globulin" fraction a further precipitate is formed between 70 to 75 per cent saturation. This carries the toxin, which also appears to be a protein derivative, since it is not dissolved or inactivated by lipoid solvents, is destroyed by heating to 90° for one hour, and is rendered inactive by digestion with trypsin.

A suggestive analogy has been drawn by Hallauer (12) between the reversible inactivation of dysentery toxin by dilute acids and the reversible ring and open chain tautomerism observed by Bergmann and Miekeley (3a) in the case of certain amino acid derivatives. These changes, taking benzoylserine as an example, may be pictured as follows:

$$
\begin{array}{ccc}
& \begin{array}{l} CH_2\!-\!O \\ \quad | \qquad\quad \searrow \!\!C\cdot C_6H_5 \\ CH\!-\!N\!\!\nearrow \\ \quad | \\ COOH \end{array} & \\[2ex]
\text{alkali}\nearrow & & \searrow \text{acid} \\[2ex]
\begin{array}{l} CH_2OH \\ | \\ CHNHCOC_6H_5 \\ | \\ COOH \end{array} & \xleftarrow{\text{alkali}} & \begin{array}{l} CH_2\!-\!O\!-\!COC_6H_5 \\ | \\ CHNH_2 \\ | \\ COOH \end{array}
\end{array}
$$

Hallauer suggested that some such intramolecular rearrangement occurs in the detoxification and loss of antitoxin-producing power of dysentery toxin when exposed to the action of dilute hydrochloric acid, and in the recovery of both functions on addition of alkali.

C. *Bacterial proteins.* The specific properties of pneumococcus "nucleoprotein," as contrasted with those of the polysaccharides of this microörganism, will be discussed under the section on Carbohydrates.

As a result of a series of studies on the active principle of tuberculin, Long and Seibert (13) present new evidence for the view that the substance producing the skin reaction is a protein (cf. also Mueller (14)). The active material, isolated after growth of the tubercle bacillus on a protein-free medium, was completely precipitated by three-fourths saturation with ammonium sulfate and was separable into a water-soluble, heat coagulable protein, a water-insoluble, non-coagulable protein, and a water soluble protein which was not coagulated by heat. The last fraction had the highest potency and is believed to be a whole protein, or one hardly degraded as far as the proteose stage, since it is attacked, with loss of activity, by pepsin and acid, or trypsin and alkali, but not by trypsin in neutral solution, or by erepsin. More recently Seibert (15) has isolated a crystalline protein from this fraction, but was uncertain whether or not this represented the true active substance of tuberculin.

D. *Antibodies.* The actual chemical nature of antibodies is still a matter of doubt. The question is as yet unanswered whether antibodies are themselves proteins, or whether they are substances of unknown constitution, so firmly attached to one or another fraction of the proteins of immune serum that separation from these has been impossible.

Since Salkowski's brief report on protein-free diphtheria antitoxin solutions (16) was shown to be in error (17) the work of Huntoon and his collaborators (18) and of Ottenberg and Stenbuck (19) has remained the chief recent evidence in favor of the non-protein nature of antibodies.

The former group, in extending the work of earlier investigators,[1] absorbed the antibodies from antipneumococcus serum with homologous pneumococci, washed the agglutinated organisms, and found a considerable proportion of the antigen-antibody complex to be dissociated at 42 to 55° by mildly alkaline solutions or those

[1] For bibliography cf. (18); cf. also Gay and Chickering, Journ. Exper. Med., 1915, xxi, 389; Chickering, Ibid., 1915, xxii, 248.

containing sugar. The extracts contained traces of protein, but since a portion of this could be eliminated by precipitation on dialysis or by acetic acid without great loss of potency, and since the antibodies appeared resistant to trypsin and did not correspond in solubility to typical euglobulin or pseudoglobulin, it was concluded that they differed from ordinary serum proteins. Ottenberg and Stenbuck found that the agglutinins in typhoid antibody solutions obtained by Huntoon's method could be largely precipitated at about pH 6.4 by small amounts of copper ion. After two reprecipitations by solution in dilute acid and readjustment to the proper pH the antibody solutions no longer gave the usual protein reactions. The final solution contained about one-twentieth of the agglutinin originally present in the serum, but only 1/3450 of the nitrogen. Less favorable results were obtained with the pneumococcus.

In considering this work it must be borne in mind that solutions obtained by the dissociation of sensitized bacteria are extremely dilute, and that the ordinary tests for protein are far from sensitive. It would therefore require the concentration of large amounts of material and the study of the actually isolated antibody substance, both chemically and immunologically, before it could be stated with certainty whether or not a protein-free antibody had been attained by any of these methods.

On the other hand, there are data pointing toward the actual protein nature of antibodies. Locke and Hirsh (20), later with Main (21), fractionated anti-sheep rabbit hemolysin both by electrodialysis and by absorption on sheep cells, dissociation of the antibody-antigen complex with ether, and extraction of the hemolysin with 0.001 N sulfuric acid. The hemolysin which separated on neutralization still contained some globulin derived from the cells used for absorption, but appeared to consist entirely of protein of the general behavior of the globulins. After further purification by electrodialysis and isoelectric flocculation it still maintained its protein characteristics, although in the best preparations one hemolytic unit was associated with as little as 0.000,044 mgm. of protein.

In Felton's extensive work on the purification of pneumococcus antibodies (22) there is also much to indicate the actual protein nature of antibodies. Felton found that when antipneumococcus serum is diluted with 15 to 20 volumes of slightly acidulated water or weak buffer solution a large proportion of the antibodies is usually precipitated and about 90 per cent of the serum proteins remain in solution.

This method is undoubtedly the simplest yet proposed for effecting a rapid and far-reaching initial purification of pneumococcus antibodies. Further fractionation without extensive losses was difficult, but by removing the small portion separating on dilution with two volumes of water, and also discarding the relatively inactive first and last fractions obtained on gradual saturation with ammonium sulfate, Felton recovered a highly potent so-called pseudoglobulin fraction with properties differing from the corresponding portion of normal serum. The chief differences hitherto observed were the shape of the titration curve of the antibody-containing globulin and its isoelectric point at about pH 6.8, a reaction considerably more alkaline than that of the isoelectric points of the ordinary serum globulins.

Felton, of course, does not consider this protein fraction to be pure antibody. Indeed, the writer has found as much as 60 to 75 per cent of the protein in the initial solutions obtained by Felton's method to remain unprecipitated by the appropriate soluble specific substance.[2] What is significant, however, is, that on further purification and removal of a portion of the inactive material Felton appears to have obtained a modified globulin. These modifications could scarcely have been induced by the cautious handling of the material, and if they are confirmed by the finding of other chemical changes caused by the presence of increasing amounts of actual antibody substance, it would become very difficult to believe that antibodies were other than proteins appropriately modified.

II. LIPOIDS. *A. Heterogenetic antigens.* It will be recalled that Forssman (23) originally showed that following the injection of certain animal tissues of unrelated species common hemolytic antibodies for sheep corpuscles appear, and that Landsteiner (24) and Taniguchi (25) demonstrated such heterogenetic antigens to consist of two component parts, one a protein, the other probably a lipoid. It was also shown by Landsteiner and Simms (26) that the lipoid fraction of the heterogenetic antigen, which is itself practically devoid of antigenic properties, acquires true antigenicity when combined with protein, and that the antibodies thus induced react with the lipoid fraction. The protein or serum with which the lipoid was combined made no difference in the specificity of the resulting antibodies for the heterogenetic lipoid fraction, thus showing this fraction to be the actual specific factor in the formation of the antibodies. Further work has

---

[2] Unpublished results. Felton found 46 per cent in an apparently more highly purified solution.

since been done on this lipoid fraction with a view to separating the actual specific substance, or "haptene," as Landsteiner has termed the non-antigenic, specifically reacting portion of complex antigens (24).

After extensive purification by fractional precipitation from various solvents and by precipitation with basic lead acetate, Sordelli, Wernicke and Deulofeu (27) concluded that the active material was not in the cerebroside fraction, contrary to earlier views. Landsteiner and Levene (28) reached the same conclusions, but found the activity of their purified preparations to be enhanced by the addition of crude sphyngomyelin, in itself practically inactive. By means of a prolonged series of fractionations from a number of solvents they obtained material showing $[\alpha]_D + 20°$ in water. This water-soluble product was composed both of lipoid and carbohydrate, since on hydrolysis it yielded soluble reducing sugars which gave purple and green colors with orcin, hydrochloric acid and copper sulfate, and also formed osazones. An insoluble portion was precipitated during the hydrolysis, and from this higher fatty acids and a base resembling sphingosine were obtained.

A product with somewhat similar solubilities and also yielding sugars and fatty acids on hydrolysis has been isolated by Dienes and Schönheit (29) by fractionation of the lipoids of the tubercle bacillus after preliminary extraction with acetone. This product, which separated from a dry ether solution of a partially purified specific fraction on long standing, showed complement fixation with rabbit antiserum to the same strain of tubercle bacillus at a dilution of about 1:10,000,000. It contained both nitrogen and phosphorus and also precipitated the immune serum at a dilution of 1:1,000,000 (30) (cf. also p. 122), so that it was possibly not a single substance.

As far as can be determined from the use of crude alcoholic extracts it is also possibly a lipoidal fraction of the red blood cells which is responsible for the specific differentiation of blood groups among human beings (31).

A new significance is thus given to lipoids as immune substances. Although Schmidt (32) has reported the lipoids of the typhoid group to possess no antigenic or specific properties, and although there is no convincing evidence that pure, isolated lipoids can act antigenically, it requires apparently but the loosest kind of combination with protein to convert lipoids into antigens capable of producing a specific antibody response. Whether the production of specific antibodies is due entirely to the lipoid fraction of the complex material which has been isolated, for example, from the specific part

of the heterogenetic antigen, or is due in part to the carbohydrate fraction, would seem still to be an open question in view of the findings discussed in the next section.

The application of the above results to the Wassermann reaction was a quick development, and has been extensively discussed by Sachs and his co-workers (33), who believe that spirochete protein is especially active in converting tissue lipoid into an antigen. However, the interpretation of their findings has been questioned (34), so that the true nature of the Wassermann reaction is still a matter of doubt.

III. CARBOHYDRATES. Recent work has shown that bacterial polysaccharides, as well as bacterial protein, influence largely the antigenic character of a number of microörganisms although these sugar derivatives are not in themselves antigenic when isolated in a state of comparative purity. The chemistry and immunological significance of these carbohydrates are therefore discussed below in considerable detail, since on the one hand many puzzling phenomena of bacterial specificity and interrelationship have been made clear, and on the other, a new field of sugar chemistry would appear to have been opened up.

After the demonstration of the existence of three fixed antigenic types of Pneumococcus (35), Dochez and Avery discovered in fluid cultures of types I, II and III of this microbe the existence of a substance which precipitated specifically in antipneumococcus serum of the homologous type (36). This "soluble specific substance" was also found in the body fluids of infected animals and was even demonstrated in the urine from approximately two-thirds of the cases suffering from pneumonia due to pneumococci of types I, II and III. Subsequently, from filtered alkaline extracts of several varieties of bacteria, including pneumococci, Zinsser and Parker (37) prepared products which they called "residue antigens," which appeared free from coagulable protein, were specifically precipitable by homologous antisera, were acid- and heat-resistant, and were considered analogous to the previously described soluble specific substance of Pneumococcus.

Since Dochez and Avery had found the specific substance to be thermostable, resistant to the action of trypsin and many reagents, and type specific in the highest degree, it seemed an ideal basis for the beginning of a study of the relation between bacterial specificity and chemical constitution, and this was accordingly undertaken (38).

The type II pneumococcus was first studied and the fractionation and purification of the specific substance were followed at each step

by means of the precipitin test. Eight-day cultures of the organism in meat-infusion phosphate broth were concentrated on the water-bath to about one-fifteenth volume, precipitated with 1.2 volumes of alcohol, and centrifuged. Of the three layers formed, the middle gummy layer contained most of the specific substance, and a large proportion of the accompanying impurities was thus eliminated in a single step. Further purification of the middle layers from about 300 liters of culture at a time depended upon repeated precipitation with alcohol under varying conditions, also by means of saturation with ammonium sulfate, and final precipitation by pouring into 10 to 15 volumes of acetone. The yield was usually from 2.5 to 3.5 grams. As the purification proceeded the material isolated took on more and more the properties of a polysaccharide, so that it became evident that a sugar derivative was at least the carrier of whatever might be the true specific substance itself. Attempts were made to separate this hypothetical substance from the polysaccharide by precipitation with basic lead acetate, uranyl nitrate, or safranine, by adsorption on alumina and recovery from this, and even by specific precipitation with a large quantity of type II antibody solution (39) and recovery of the specific substance from the immune precipitate, but these failed to effect a significant change in properties, even when pneumococci themselves were used as starting material instead of the broth culture. Attempts at a separation by means of carbohydrate splitting enzymes also failed as the sugar derivative proved resistant to this type of hydrolysis. Moreover, when exposed to the action of 1:1 hydrochloric acid in the cold, the substance diminished in specific activity only after reducing sugars began to appear, so that the specific substance and the polysaccharide, if not identical, appeared at least to be very closely associated. On hydrolysis the specific product yielded about 70 per cent of reducing sugars consisting mainly of glucose, as shown by the isolation of glucosazone and the formation of saccharic acid on oxidation. Other possible constituents remained unidentified.

Little work was necessary on the soluble specific substance of type III pneumococcus to show that marked chemical differences existed between it and the corresponding derivative of type II. The type III substance proved to be the soluble salt of an insoluble acid, far stronger than the type II substance, and capable of being thrown out of solution by an excess of strong hydrochloric acid. This property was of great use, not only in separating the specific substance from accompanying glycogen or erythrodextrin, but also in effecting a

rapid purification without the use of ammonium sulfate and with
fewer fractionations by alcohol. Successive lots also agreed very
closely in their physical and chemical properties, indicating that one
was apparently dealing in this case with a much more definite chemical
entity than was the type II product.

The type III soluble specific substance was thus also isolated as a
nitrogen-free polysaccharide. No further purification could be realized
either by precipitation with barium hydroxide in excess, or by adsorp-
tion on highly active alumina (40).

Once the properties of the type III specific substance had been
established it was considered proper to add glucose to the usual Pneu-
mococcus broth, thus securing greatly increased growth and improving
the yields of specific substance from 6 to 9 grams per 300 liters of
broth to 35 to 40 grams. With these increased amounts of material
a more detailed chemical study of the substance was made possible.

When the type III specific substance is treated in the cold with
75 per cent (by weight) sulfuric acid until it no longer precipitates
on dilution, and is then further hydrolyzed with boiling normal sulfuric
acid until the maximum reducing power is attained, a small amount
of glucose is formed. This was identified through the osazone and
by oxidation to saccharic acid. 85 per cent of the hydrolysis products
are, however, precipitated by basic lead acetate and yield a crystalline
morphine salt, from which is obtained a sugar acid differing little from
the crude acid before purification. Its reducing power is 50 per cent
that of glucose; its acid equivalent is 363, while the value calculated
for a disaccharide acid, $C_{11}H_{19}O_{10} \cdot COOH$, is 356; on prolonged hy-
drolysis only a small amount of glucose, in addition to unhydrolyzed
material could be isolated, the acid half of the portion hydrolyzed
apparently decomposing similarly to glucuronic acid and yielding
approximately the expected amount of furfural; the reducing group of
this disaccharide acid is aldehydic, as shown by its quantitative de-
termination by the Willstätter-Schudel method (41); the acid also
gives the color reaction with naphthoresorcinol characteristic of the
glucuronic acid type. Whether the union of the glucose to the sugar
acid is through the reducing group of the glucose, or through that of
the sugar acid is as yet unknown.

Since only about 9.5 per cent of the hydrolysis products of the original
polysaccharide appear as glucose, and since the above disaccharide
or aldobionic acid has been shown to yield glucose slowly on further
hydrolysis, it is not illogical to assume that the glucose set free during

the hydrolysis of the specific polysaccharide owes its origin, not to a separate part of the carbohydrate molecule, but chiefly to a secondary reaction involving the aldobionic acid. This is also supported by the fact that no glucose is split off during the preliminary hydrolysis by 75 per cent sulfuric acid in the cold. Since also, the actual partial hydrolysis products obtained in this way show, by their acid equivalents, one carboxyl group for every two sugar nuclei, it would seem that the polysaccharide as a whole is built up of units of the disaccharide acid. A substance of this composition should have the formula $(C_{12}H_{18}O_{11})_n$, with an acid equivalent of 338 and a carbon and hydrogen content of 42.6 per cent and 5.4 per cent, respectively. These figures are practicaly identical with the values obtained on analysis of the substance (cf. table 1).

The soluble specific substance of type I pneumococcus was present in the culture fluid in relatively smaller amount than those of the type II and type III organisms and therefore required 1.3 to 1.4 volumes of alcohol for its precipitation from the culture concentrate. Other modifications were necessitated by the insolubility of the substance at its isoelectric point, about pH 4, and advantage was taken of its ability to form a precipitate with barium hydroxide in excess. Finally the specific substance was reprecipitated by alcohol in the presence of hydrochloric acid, and dialyzed. Being a weak base, it precipitated as the excess of hydrochloric acid was removed. The yield was 2 to 3 grams. A preparation from type I pneumococci which had been separated from the accompanying broth, and one subjected to additional purification by adsorption on alumina failed to show differences from those isolated as above.

The type I soluble specific substance also appears to be a sugar derivative, but differs sharply from the other two substances in the lower percentage of sugar liberated on hydrolysis and in containing nitrogen as an apparently essential component.

The following data and table 1 are given as a brief summary of the properties of the soluble specific substances of the three fixed types of pneumococcus in the state of purity thus far attained.

The type I soluble specific substance rotates the plane of polarized light about 300° to the right, is a strong acid and a weak base, and is very sparingly soluble at its isoelectric point, which lies at about pH 4. In spite of a nitrogen content of 5.0 per cent the substance gives none of the usual protein color tests. One-half of the nitrogen is liberated on treatment with nitrous acid and reducing sugars appear at the

same time, while the specific reaction vanishes. Under the same conditions the type II and type III substances are unaffected. It would thus appear that this portion of the nitrogen, at least, is an integral part of the specific substance, and is possibly linked to the reducing group of a sugar derivative, as Karrer believes is the case in the polyglucosamines (42). The substance gives the color reaction for glucuronic acid with naphthoresorcinol, but yields mucic acid on oxidation, indicating a relationship to galactose. Since the carbon and hydrogen contents of the substance are close to the theoretical values for polysaccharides it appears possible that in it a nitrogenous sugar derivative is linked to galacturonic acid through the reducing group of the latter. The type I substance is precipitated by barium hydroxide in excess, by heavy metal salts, and by phosphotungstic acid. In the specific precipitin reaction with type I antipneumococcus serum it can be detected in dilutions as great as 1:6,000,000, while at a concentration of 1:400 it gives a faint cloud with type III antiserum.

The soluble specific substance of type II pneumococcus is apparently a weakly acidic, nitrogen-free polysaccharide made up chiefly of glucose units. Its acid equivalent is about 1250 and the specific optical rotation is about +74°. It is not precipitated by barium hydroxide or heavy metal salts with the exception of basic lead acetate and uranyl compounds. It reacts at a dilution of 1:5,000,000 with type II antipneumococcus serum but does not precipitate type I and type III antisera at a concentration of 1:400. The substance is converted by acetic anhydride and pyridine into a very sparingly soluble triacetyl derivative.

The type III soluble substance, while also isolated as a nitrogen-free polysaccharide, is a strong acid with an acid equivalent of about 340. It rotates the plane of polarized light about 33° to the left. It is precipitated by barium hydroxide in excess and by heavy metal salts, and is also rendered insoluble by the addition of strong hydrochloric acid. In as high a dilution as 1:6,000,000 it still reacts with type III antipneumococcus serum. It appears to be a definite chemical individual, built up by condensation of an aldobionic acid in such a way that the reducing groups disappear and the carboxyl groups remain free. The evidence so far collected indicates that in the aldobionic acid, $C_{12}H_{20}O_{12}$, glucose and a hexose-uronic acid are combined in such a way that one aldehydic group and the carboxyl remain free. The polysaccharide is thus unusual not only in its possession of the

property of immunological specificity, but in its chemical constitution as well.

The three polysaccharides contain no sulfur or phosphorus and differ from the starch-glycogen group of carbohydrates in their acid properties, in giving no color with iodine, and in their resistance to the ordinary carbohydrate-splitting enzymes. Each substance breaks

TABLE 1

Soluble specific substances of the three fixed antigenic types of pneumococcus and of Friedländer bacillus (type B)

TYPE	$[\alpha]_D$	ACID EQUIVALENT	C	H	N	REDUCING SUGARS ON HYDROLYSIS		HIGHEST DILUTION GIVING PRECIPITATE WITH HOMOLOGOUS IMMUNE SERUM
			per cent	*per cent*	*per cent*	*Calculated as glucose*		
I	+300°		43.3*	5.8	5.0†	28	(Galacturonic acid) (Amino sugar derivative)	1:6,000,000
II	+74°	1250	45.8	6.4	0.0	70	Glucose	1:5,000,000
III	−33°	340	42.7	5.3	0.0	75	Aldobionic acid, glucose	1:6,000,000
Friedländer bacillus B	+100°	685	44.6	6.1	0.0	73	Glucose	1:2,000,000‡

* Theory for $(C_6H_{10}O_5)_n$, C. 44.4 per cent; H, 6.2 per cent.

† Amino N, 2.5 per cent.

‡ Rabbit antiserum.

down on hydrolysis into reducing sugars, a part of which, at least, is peculiar to itself. The type I substance differs sharply from the other two in containing nitrogen and in possessing basic as well as acid properties, while of the other two substances, the type II is a dextrorotatory weak acid and the type III a levorotatory strong acid. Especially striking is the occurrence of specific substances of such widely differing properties in microorganisms as closely related as the three fixed types of Pneumococcus.

With the possible exception of the type III polysaccharide, it is

doubtful whether each of the specific substances as at present isolated represents a definite chemical compound. However, in the case of the three fixed types of Pneumococcus three totally distinct carbohydrates have been isolated from cultures grown in the same medium. Successive preparations of each specific substance have been quite uniform regardless of the widely different methods employed in the process of purification, and whether derived from the microorganisms themselves or from autolyzed broth cultures. Furthermore, the only one of these substances as yet investigated in detail appears to differ in its structure from that of any other known non-nitrogenous polysaccharide.

It is thought that these and other considerations based on the data presented warrant the belief that the three polysaccharides isolated represent the actual specific substances, stripped of at least a large portion of accompanying impurities, and that they do not merely represent inert material carrying an extremely minute amount of the true specific compounds. This, if admitted, would afford a chemical corroboration of the fact of type specificity in Pneumococcus, since only biological methods had been used in the original investigation (35).

The extension of the above methods to the Friedländer group of bacilli resulted in the preparation of another polysaccharide with specific properties (43), from agar cultures of the so-called "E" strain of this bacillus (now known as type B (44)). After repeated precipitation with alcohol, then with barium hydroxide, and finally with alcohol in the presence of hydrochloric acid, it was obtained as a nitrogen-free strong acid with an equivalent value of about 685. The specific optical rotation was $+100°$. It did not precipitate with silver or copper ions, but was thrown down by barium hydroxide in excess and by both neutral and basic lead acetates. It gave no color with iodine. The polysaccharide itself was non-reducing but on hydrolysis with mineral acid yielded reducing sugars, among which glucose was shown to be present by isolation of its osazone and by oxidation to saccharic acid.

From table 1 it will be seen that this substance greatly resembles that of the type II pneumococcus, and the similarity was found to extend even to precipitation of type II antipneumococcus serum. The type II substance in its present state of purity is, however, a weaker acid and is not precipitated by barium hydroxide or neutral lead acetate. It is possible that these differences depend only on the presence of different impurities in the two cases, but opposed to this view are the

findings that different preparations of each substance, purified by widely different methods, showed great constancy in each case, and that the absorption of agglutinins and precipitins is not reciprocal with the two organisms. If the fact that bacteria possess mutual absorptive capacity be accepted as the criterion of their antigenic identity, then the failure of the organisms in question to exhibit this property may be taken as further evidence of the lack of identity of the substances involved.

However, granted a chemical difference between the two specific substances, it becomes necessary to account for their marked immunological similarity. In the absence of further evidence as to the structural relations of the two polysaccharides it seems reasonable to assume that both contain in a portion of the complex molecule the same or a closely similar configuration of atoms. This essential similarity in molecular grouping would then determine the immunological similarity of the two substances.

In the case of Pneumococcus it has been shown that the polysaccharides by themselves are not antigenic, and it is believed that they become antigenic only when attached to some other substance, possibly the protein of the cell. The type-specific character of the antigenic response, however, is dependent almost entirely upon the nature of the polysaccharide and not upon the substance to which it is attached. Therefore, since the specific carbohydrate of the Friedländer bacillus (type B) and that of type II pneumococcus exhibit similar chemical properties the antigenic response to each may also be similar even though the proteins or other substances with which they are combined be quite dissimilar.

A striking and probably analogous example of common antigenic properties in substances of remote biological origin is furnished by the phenomenon of heterogenetic specificity originally described by Forssman (23; cf. also p. 112).

The fact that two biologically unrelated organisms, pneumococcus type II and Friedländer's bacillus (type B), possess certain similar serological and antigenic properties suggests that examples of heterogenetic specificity likewise occur among bacteria. In the case of the bacteria hitherto studied, however, the specific substance appears to be a polysaccharide, instead of a lipoid, and it further appears probable that when the analogous specific polysaccharides of otherwise totally unrelated microörganisms correspond sufficiently in chemical constitution an immunological correspondence also results.

From another strain of Friedländer's bacillus, the type of which has not yet been identified, Mueller, Smith and Litarczek (45) isolated carbohydrate-containing material with a nitrogen content of 1.3 per cent and showed that at high dilutions it precipitated homologous immune serum.

An apparent polysaccharide with specific properties has also been discovered in the tubercle bacillus, and somewhat similar products have been obtained by Laidlaw and Dudley (46) from the defatted bacilli, and by Mueller (47) from the culture itself, by widely differing methods. Laidlaw and Dudley heated the bacilli with weak alkali, and the portion of the extract not precipitated by acetic acid or copper sulfate was adsorbed with uranium hydroxide and the specific material carried by the adsorbent freed from this and separated from accompanying glycogen by repeated fractionation with alcohol. Mueller freed the culture concentrate from nucleic acid derivatives, treated it with "antiformin," and fractionated the specific material under varying conditions from alcohol. In both instances products giving the reactions of a pentose-containing polysaccharide were obtained, dextrorotatory, practically nitrogen free, and reacting at high dilutions with the sera of animals immunized against the tubercle bacillus. The sugars obtained on hydrolysis were not identified. Laidlaw and Dudley's material showed $[\alpha]_{Hg\ green} + 79°$ and 50 per cent of reducing sugars on hydrolysis, while the figures for Mueller's product were $[\alpha]_D +24°$ and 99 per cent, respectively. The polysaccharide appears responsible for the precipitin reaction given by tuberculin.

Specifically reacting material, also apparently carbohydrate in nature, has been isolated from yeast by fractionation with alcohol. The product, closely associated with, or identical with the yeast gum, precipitated the sera of rabbits immunized against the yeast (48).

The wide distribution of specific polysaccharides among microorganisms of the most diverse types made it seem not improbable that there might occur among the higher plant forms other carbohydrates with specific properties. In a series of tests on water-soluble plant gums samples of gum arabic (gum acacia) were actually found which showed some degree of specific activity. By fractional hydrolysis with 1:1 hydrochloric acid it was found possible to remove about one-half of the pentose present. $[\alpha]_D$ of the recovered gum was about $-10°$ and its specific activity had increased 100- to 150-fold (49). The interpretation of these findings is withheld until further data are available.

Initial studies of the relationship of the soluble specific substances of bacterial origin to other cell constituents have been made in the case of Pneumococcus (38b, 50, 51, 38e). When these microbes are dissolved, either with the aid of bile, or by repeated freezing and thawing, the resulting solution yields a precipitate of so-called "nucleoprotein" on acidification with acetic acid. While probably a mixture consisting largely of nucleoprotein and mucoid, it still possesses immunological properties which differ sharply from those of the soluble specific substance. In the first place, the protein is antigenic, while the soluble specific substance, though reacting specifically with antibodies to the highest degree, is non-antigenic and unable by itself to stimulate the production of antibodies when injected into animals. Moreover, the protein isolated from any one of the three fixed types of Pneumococcus, or from a strain of the heterogeneous group IV, appears serologically the same as that from any of the other types. Thus this portion of the pneumococcus protein is not type-specific, like the soluble specific substance, but is, rather, species-specific.

Since many of the immune reactions participated in by bacteria are presumably surface phenomena, the nature of the reactive material at the periphery of the cell may determine the readiness of response and even the specificity of reaction. Pneumococcus is an encapsulated organism, and there are grounds for the belief that the ectoplasmic layer of the cell is composed of carbohydrate material which is identical in all its biological characters with the type-specific substance. On the other hand, the endoplasm, or somatic substance, consists largely of protein which, as previously pointed out, is species- and not type-specific. This protein is possessed in common by all pneumococci, while the carbohydrate is chemically distinct and serologically specific for each of the three fixed types. The cell, therefore, may be conceived of as so constituted that there is disposed at its periphery a highly reactive substance upon which type specificity depends.

The fact that this specifically reactive carbohydrate is non-antigenic when separated from the other cellular constituents and is capable of inciting antibody formation only in the form in which it is present in the intact cell, forces the conclusion that in the latter instance it exists not merely as free carbohydrate but also in combination with some other substance which confers upon it specific antigenic properties.

Immunization with intact bacteria containing this carbohydrate complex elicits antibodies which not only agglutinate the formed cells but precipitate solutions of the carbohydrate isolated from pneumococci

against S alone or against both S and P separately, depending upon whether or not these suspensions contain only intact cells or a mixture of both intact and dissolved cell bodies. Since pneumococci readily undergo autolysis and dissolution, suspensions and indeed cultures of these organisms almost invariably contain not only formed elements, but also more or less of dissociated cell constituents in solution. Therefore, use of suspensions of pneumococci containing both intact cells and the soluble products of cell disintegration yields on immunization not only type-specific antibodies but antibodies reacting with the protein substance which is common to all pneumococci. While the former generally predominate it is the presence of this protein antibody with its broader zone of activity which is responsible for the confusing cross-immunity reactions occasionally encountered in supposedly type-specific sera and which has in some instances led workers even to deny the existence of three distinct antigenic types of pneumococci. That the two sets of antibodies involved are separate and distinct is shown by absorption tests; the antiprotein reacting bodies in such sera can be removed by absorption with the protein of a heterologous type without diminishing the titer of specific agglutinins for the homologous culture or the precipitins for the specific polysaccharide of the corresponding type.

The possible relation of the specific polysaccharides to virulence and disease has been discussed elsewhere (loc. cit., also 52, 53), and is outside the scope of the present paper, as is also a discussion of advances which the above type of analysis has already permitted in the study of the Encapsulating group (54) and the non-hemolytic streptococci (55).

The specifically reacting polysaccharides offer much of chemical interest. While it had generally been assumed that only the proteins and their derivatives provided the innumerable opportunities for isomerism and subtle changes requisite for the substances exhibiting the phenomena of specificity, the discovery of carbohydrates with specific properties is not as surprising as might appear on first thought. When one considers the number of asymmetric carbon atoms in the hexoses and pentoses, the different possible points of attachment of the lactone bridge, the possibility of α- and β- glucosidic unions at various positions in the molecule, and the addition of sugar acids, the analogs of amino acids, to the large number of sugars theoretically capable of entering into the composition of such polysaccharides, it becomes clear that perhaps only among the carbohydrates could another sufficiently large and protean group of substances be found to afford the possibility of specific manifestations.

At the present early stage of the development of the field thus opened up it would be idle to speculate on the relationship within this group of specificity to chemical constitution. That some such relation exists, however, and is reflected by marked chemical changes has been fully demonstrated in the case of the soluble specific substances of the three antigenic types of Pneumococcus. Also significant is the finding that, even in the case of such widely different microörganisms as Pneumococcus type II and the type B Friedländer bacillus, a certain similarity in the chemical nature of their specific polysaccharides is accompanied by a corresponding similarity in the immunological properties of the organisms themselves.

So far only the specific polysaccharide of type III pneumococcus has been investigated in detail, and it is too early to render judgment as to whether or not the polysaccharides with specific properties form a chemically distinct group of carbohydrates. In this one instance the structure certainly appears different from that of any known non-nitrogenous sugar derivative, although an analogy to the nitrogen-containing chondroitin sulfuric acid (56) is discernible, and perhaps even a distant relationship to the pectins (57). In this connection the finding of a specifically reacting polysaccharide as a constituent of gum arabic is of interest, and it is not impossible that sugar derivatives with specific properties may occupy a position of biological significance among higher forms of life as well as among bacteria.

Under the three main headings which have been taken up, the list of immune substances of interest and importance has by no means been exhausted. Complement, for example, has not been discussed, since recent work has failed to establish whether complement is merely a colloidal state, or an actual chemical entity or group. The study of bacteriophage, as well, is still in too early and controversial a state for discussion in a review of such limited scope as the present one.

The fragmentary and incomplete character of these gropings toward the actual chemical nature of immune substances is obvious. Future progress is dependent upon new methods as well as upon the application of those used with success in other fields of organic chemistry. However, the little that has already been accomplished has not been without theoretical and practical implications sufficient to stimulate renewed endeavor.

BIBLIOGRAPHY

(1) WELLS, H. G. The chemical aspects of immunity. New York, 1925.
(2) ABDERHALDEN, E. ET AL. Zeitschr. physiol. Chem., 1924, cxxxix, 169, 181, and numerous other papers.
(3a) BERGMANN, M. AND A. MIEKELEY. Ibid., 1924, cxl, 128.
(3b) BERGMANN, MIEKELEY, F. WEINMANN AND E. KANNS. Ibid., 1925, cxliii, 108.
(4) TROENSGAARD, N. AND Eug. FISCHER. Ibid., 1925, cxlii, 35; cxliii, 304, and other papers.
(5) HEIDELBERGER, M. AND K. LANDSTEINER. Journ. Exper. Med., 1923, xxxviii, 561.
(6) LANDSTEINER. Klin. Wochenschr. (in press).
(7) DERNBY, K. G. Journ. Gen. Physiol., 1926, viii, 311.
(8) WATSON, O. F. AND E. LANGSTAFF. Biochem. Journ., 1926, xx, 763.
(9) WATSON AND U. WALLACE. Journ. Path. Bact., 1924, xxvii, 289.
(10) GLENNY, A. T. AND G. S. WALPOLE. Biochem. Journ., 1915, ix, 298.
(11) HUNTOON, F. M. Proc. Soc. Exper. Biol. and Med., 1923–4, xxi, 513.
(12) HALLAUER, C. Zeitschr. Hyg., 1925, cv, 138.
(13) LONG, E. R. AND F. B. SEIBERT. Amer. Rev. Tuberc., 1926, xiii, 393, 398, 404, 408, 431, 441, 448.
(14) MUELLER, J. H. Journ. Exper. Med., 1926, xliii, 1.
(15) SEIBERT. Science, 1926, lxiii, 619.
(16) SALKOWSKI, E. Biochem. Zeitschr., 1922, cxxxii, 84.
(17) DOLD, H. AND E. FREUDENBERG. Ibid., 1925, clxii, 169.
(18a). HUNTOON, F. M. Journ. Immunol., 1921, vi, 117.
(18b) HUNTOON AND S. ETRIS. Ibid., vi, 123.
(18c) HUNTOON, P. MASUCCI AND E. HANNUM. Ibid., vi, 185.
(19) OTTENBERG, R. Proc. Soc. Exper. Biol. and Med., 1923–4, xxi, 14; OTTENBERG AND F. STENBUCK. Ibid., xxi, 303; 1924–5, xxii, 211, 215; 1925–6, xxiii, 23.
(20) LOCKE, A. AND E. F. HIRSCH. Journ. Inf. Dis., 1924, xxxv, 519; 1925, xxxvii, 449.
(21) LOCKE, E. R. MAIN AND HIRSCH. Ibid., 1926, xxxviii, 126.
(22a) FELTON, L. D. Boston Med. and Surg. Journ., 1924, cxc, 819.
(22b) FELTON. Journ. Inf. Dis., 1925, xxxvii, 199, 309; Bull. Johns Hopkins Hosp., 1926, xxxviii, 33.
(22c) FELTON AND G. H. BAILEY. Journ. Immunol., 1926, xi, 197.
(22d) Paper read before American Chemical Society, Philadelphia, September 9, 1926.
(23) FORSSMAN, J. Biochem. Zeitschr., 1911, xxxvii, 78.
(24) LANDSTEINER. Ibid., 1921, cxix, 296.
(25) TANIGUCHI, T. Journ. Path. and Bact., 1921, xxiv, 253, 254.
(26) LANDSTEINER AND H. S. SIMMS. Journ. Exper. Med., 1923, xxxviii, 127.
(27) SORDELLI, A., R. WERNICKE AND V. DEULOFEU. Compt. rend. soc. biol., 1925, xcii, 898.
(28) LANDSTEINER AND P. A. LEVENE. Journ. Immunol., 1925, x, 731; Proc. Soc. Exper. Biol. and Med., 1925–6, xxiii, 343.

(29) DIENES, L. AND E. W. SCHOENHEIT. Journ. Immunol., 1925, x, 631; Proc. Soc. Exper. Biol. and Med., 1925-6, xxiii, 106.

(30) DIENES AND J. FREUND. Journ. Immunol., 1926, xii, 137.

(31) LANDSTEINER, J. VAN DER SCHEER AND D. H. WITT. Proc. Soc. Exper. Biol. and Med., 1924-5, xxii, 289.

(32) SCHMIDT, H. Zeitschr. Immunitätsf., 1924, xxxviii, 511.

(33) SACHS, H., A. KLOPSTOCK AND A. J. WEILL. Deutsch. Med. Wochschr., 1925, li, 589, 1017.

(34a) KLOPSTOCK, F. Ibid., 1926, lii, 226.

(34b) LANDSTEINER AND VAN DER SCHEER. Proc. Soc. Exper. Biol. and Med., 1925-6, xxiii, 641.

(35) DOCHEZ, A. R. AND L. J. GILLESPIE. Journ. Amer. Med. Assoc., 1913, lxi, 727.

(36) DOCHEZ AND O. T. AVERY. Journ. Exper. Med., 1917, xxvi, 477.

(37) ZINSSER, H. AND J. T. PARKER. Ibid., 1923, xxxvii, 275.

(38a) HEIDELBERGER AND AVERY. Ibid., 1923, xxxviii, 73.

(38b) AVERY AND HEIDELBERGER. Ibid., 1923, xxxviii, 81.

(38c) HEIDELBERGER AND AVERY. Ibid., 1924, xl, 301.

(38d) AVERY AND HEIDELBERGER. Ibid., 1925, xlii, 367.

(38e) HEIDELBERGER, W. F. GOEBEL AND AVERY. Ibid., 1925, xlii, 727.

(38f) HEIDELBERGER AND GOEBEL. Journ. Biol. Chem., 1926, lxx, 613.

(39) Prepared essentially according to FELTON (22a).

(40) Prepared according to R. WILLSTÄTTER AND H. KRAUT. Ber., 1923, lvi, 149.

(41) WILLSTÄTTER AND C. SCHUDEL. Ber., 1918, li, 780.

(42) KARRER, P., O. SCHNIDER AND A. P. SMIRNOFF. Helvetica Chim. Acta, 1924, vii, 1039.

(43a) HEIDELBERGER, GOEBEL AND AVERY. Journ. Exper. Med., 1925, xlii, 701.

(43b) AVERY, HEIDELBERGER AND GOEBEL. Ibid., 1925, xlii, 709.

(44) JULIANELLE, L. A. Ibid., 1926, xliv, 113.

(45) MUELLER, D. E. SMITH AND S. LITARCZEK. Proc. Soc. Exper. Biol. and Med., 1924-5, xxii, 373.

(46) LAIDLAW, P. P. AND H. W. DUDLEY. Brit. Journ. Exper. Path., 1925, vi, 197.

(47) MUELLER. Journ. Exper. Med., 1926, xliii, 9.

(48) MUELLER AND J. TOMCSIK. Ibid., 1924, xl, 343.

(49) HEIDELBERGER, AVERY AND GOEBEL. Paper read before Annual Meeting, American Chemical Society, Philadelphia, September 9, 1926.

(50) ZINSSER AND T. TAMIYA. Journ. Exper. Med., 1925, xlii, 311.

(51a) AVERY AND H. J. MORGAN. Ibid., 1925, xlii, 347.

(51b) AVERY AND J. M. NEILL. Ibid., 1925, xlii, 355.

(52) SIA, R. H. P. Ibid., 1926, xliii, 633.

(53) FELTON AND BAILEY. Journ. Inf. Dis., 1926, xxxviii, 131.

(54) See (44) and Journ. Exper. Med., 1926, xliv, 683, 735.

(55) LANCEFIELD, R. C. Ibid., 1925, lxii, 377.

(56 LEVENE. Hexosamines and mucoproteins. Longmans, Green & Co., London, 1925.

(57) EHRLICH, F. AND R. VON SOMMERFELD. Biochem. Zeitschr., 1926, clxviii, 263.